HPLC detection of fetal blood in meconium: improved sensitivity compared with qualitative methods

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We describe an HPLC-based method for the detection and quantification of fetal hemoglobin in stools of newborns. The new procedure is an alternative to the classic qualitative test for adult hemoglobin in meconium based on the differential stability of hemoglobin species in dilute base (Apt test). The HPLC method, based on a commercial device for hemoglobin characterization (Bio-Rad Variant), readily separates fetal and adult hemoglobin from non-hemoglobin components of meconium. To validate the method, blood and meconium were mixed in various proportions and then prepared for analysis with extraction in saline. The HPLC method accurately identified hemoglobin species even when the blood constituted only 0.5 mL per 100 g of the meconium specimen, and nearly quantitative recovery of hemoglobin was obtained at a blood content of 20 mL per 100 g of the meconium. Analysis time was 6.5 min, and preparation of sample was simple. HPLC detection of fetal blood in stools or other specimens markedly improves detection/characterization of blood in meconium.

The Apt test is a qualitative test to identify the source of blood present in stool of newborns for differential diagnosis of gastrointestinal bleeding (1). The principle of the test is based on the different susceptibilities of hemoglobin A (HbA, the major component of adult hemoglobin) and hemoglobin F (HbF, the major component of fetal/newborn hemoglobin) to alkaline denaturation. The latter is more resistant to alkaline denaturation than the former; therefore, samples containing visually detectable HbF retain a pink color, whereas samples containing HbA fade to a yellowish color after alkaline treatment. Because newborn blood contains 65–90% HbF and adult blood usually contains <1%, knowledge of whether HbF is present differentiates between newborn gastrointestinal bleeding and a maternal source of the blood (2). In general, grossly bloody stool specimens of newborns are required for accurate analysis because the outcome of the test is determined visually; the requirement for visible blood limits sensitivity, and the subjective nature of detection introduces operator-dependent variation.

To overcome these problems, we developed an HPLC method to identify the species of hemoglobin in stool specimens. We utilized the Variant HPLC system (Bio-Rad Corp.), which separates hemoglobin species by cation-exchange chromatography and quantifies the presence of each type of hemoglobin (3). Our results indicate that the HPLC method has much higher sensitivity and specificity than the Apt test.

Materials and Methods

STOOL SPECIMENS
Meconium from newborns (0–48 h of age) was pooled and stored at −20 °C until the day of the experiment. In the standard method, a weighed amount of meconium (0.05–0.08 g) was mixed with saline (9 mL of saline per gram of meconium), and the meconium was thoroughly suspended by vortex mixing. The specimen was frozen and thawed to lyse cells. After centrifugation of the mixture at 8800g for 10 min, the supernatant was used for analysis. In validation experiments, cord or adult blood was added before addition of saline to simulate a bloody stool. Paired controls for each stool specimen were prepared by substituting an equal volume of saline solution for meconium. Adult blood was drawn from healthy adults into EDTA-containing tubes; whole blood was stored at 4 °C for a maximum of 2 days before use. Cord blood was obtained as remainders of EDTA-anticoagu-

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lated specimens sent for blood typing to the hospital blood bank. Cord blood samples were stored at 4 °C for up to 48 h before use. This study was conducted in accordance with protocols approved by the Human Studies Committee of Washington University.

**APT Test**
The procedure was performed in the laboratory by two experienced technologists. A small amount of specimen was spotted onto paper, and the specimen area on the paper towel was saturated with one drop of 250 mmol/L NaOH. An immediate color change (from pink to brown) indicated the presence of HbA; persistence of a pink color indicated the presence of HbF. Adult blood and cord blood served as negative and positive controls, respectively.

**HPLC Analysis**
HPLC analysis was performed with a Bio-Rad Variant analyzer, using the manufacturer’s reagents and following the manufacturer’s protocol (β-thalassemia short program). The analyzer was programmed with a run time of 6.5 min, column temperature of 30 ± 4 °C, and two-buffer gradient elution system using sodium phosphate buffers (overall flow rate, 2.0 mL/min).

**Results**

**HPLC Detection of Hemoglobin in Meconium**
Because the HPLC method was designed for analysis of red cell lysates and the application sought was in meconium/stool, it was necessary to determine whether the method could detect HbA and HbF in the presence of meconium. Meconium contains considerable pigmented material, which absorbs at the wavelength used by the Variant (415 nm) to detect eluting hemoglobin species; when adult blood is present at 50 mL/100 g (500 μL/g) of meconium, >25% of the absorbance at that wavelength is attributable to soluble meconium pigments. A mixture of adult blood, fetal blood, and meconium was subjected to analysis after addition of saline and production of a supernatant by centrifugation. The hemoglobin fractions in the specimen were well separated by the HPLC, and non-hemoglobin components that contributed to absorbance eluted well before the hemoglobin species (Fig. 1). The chromatogram showed two major peaks with elution times corresponding to HbF and HbA, identified by retention times retained by the HPLC software and validated by analysis of adult and newborn blood specimens.

**HPLC Quantification of Hemoglobin in Meconium Specimens**
Because meconium might interfere in the measurement of hemoglobin by HPLC, we investigated the recovery of HbA and HbF in meconium-containing samples. The recovery of total hemoglobin detected in meconium samples was compared with that in paired controls where the meconium was replaced with saline. Identification of HbF required the presence of only 5 mL of cord blood in 100 g of meconium (50 μL in 1.0 g). When the ratio of cord blood to meconium was >20 mL/100 g (200 μL/g), recovery was consistently 85% of that in saline controls (Fig. 2A). Recovery of adult hemoglobin was similar to that of fetal hemoglobin (Fig. 2B).

**Hemoglobin Stability in Meconium**
Adult hemoglobin in meconium (20 mL/100 g), after addition of saline for the usual specimen processing but before centrifugation, was stable up to 8.5 h when stored at room temperature and for 24 h at 4 °C (Fig. 3A). Fetal hemoglobin in meconium was similarly stable (Fig. 3B). Addition of EDTA and/or aprotonin did not appear to improve stability (data not shown).

**Comparison of HPLC and APT Methods**
Duplicate meconium specimens with added cord blood were analyzed simultaneously with both methods. The Apt test required a minimum of 40 mL/100 g cord blood (with HbF 80% of the total hemoglobin) for detection of fetal hemoglobin (Table 1). Moreover, the reproducibility of the Apt test was very poor. Even with specimens containing 60–80 mL/100 g cord blood, detection of a positive visual endpoint was inconsistent. This inconsistency is likely caused, at least in part, by the difficulty in appreciating the red color contributed by hemoglobin in the presence of meconium, which made detection difficult with the visual Apt methodology. Generally, specimens containing <40 mL/100 g hemoglobin did not have a detectable red color to base analysis on. In contrast, the
HPLC method required only 5 mL of cord blood per 100 g of meconium to identify the hemoglobin species present reproducibly (Table 1), despite reduced quantitative recovery at this concentration.

**Precision**
Repeated analysis of a specimen with 500 µL of whole blood added (equal volumes of cord and adult blood) per gram of meconium yielded a day-to-day imprecision (CV) of 4.3% for the peak area for total hemoglobin and 3.8% for the peak area of HbF (n = 8).

**Discussion**
Here we describe the development of a new method to detect the presence of HbF in newborn meconium. Traditionally, the Apt test (1) has been used to distinguish the source of blood in such specimens for differential diagnosis of newborn gastrointestinal bleeding. Identification of HbA as the predominant hemoglobin species in the specimen indicates that the blood source is of maternal origin, likely as the result of ingestion of blood in the amnion or at birth. A finding of maternal origin for the blood can spare the newborn infant from having to...
undergo unnecessary procedures such as radiographic examinations (1). The dependence of the Apt test on subjective visual judgment for analysis yields low sensitivity and poor reproducibility, especially when the meconium specimen is not grossly bloody. Earlier investigations sought to modify the Apt test to improve the reliability of distinguishing HbA and HbF by measuring HbF-specific absorbance at 576 nm (4, 5). In one study, HbF was linearly proportional to absorbance, which allowed for quantification (4).

The HPLC method we describe here has advantages over both the original Apt test and the modified procedures based on alkaline denaturation (1, 4, 5). It is a rapid, automated, and quantitative method with high sensitivity and specificity. It eliminates the dependence on subjective visual interpretation of the Apt test and is capable of detecting fetal hemoglobin in the presence of meconium at concentrations far below the detection limit of the Apt test. The Apt test is not widely available, probably because of infrequent demand combined with the difficulty of maintaining specific reagents and proficiency for an infrequently used test. Because the HPLC test utilizes equipment and reagents already present in many laboratories and used for HbA1c or hemoglobin species analysis, maintaining the HPLC test will be much more feasible. Improved feasibility may increase the availability of this test.

Specimens prepared for HPLC analysis are sufficiently stable that analysis may be delayed for up to 24 h if permitted by the clinical circumstance. Thus, it is possible to transport specimens to a reference laboratory or another hospital for analysis when the test is not available on-site. The procedure is sufficiently rapid (preparation and analysis <1 h) to allow emergency analysis in critically ill infants. Finally, the HPLC method eliminates the need for a caustic reagent (sodium hydroxide).

The utility of hemoglobin analysis in meconium and stool may be greatly expanded by the increased sensitivity of HPLC, which will allow serial measurement to follow resolution of gastrointestinal bleeding with therapy. Moreover, it may be useful in other clinical applications to distinguish maternal and newborn blood, such as in cordocentesis sampling or in body fluids such as amniotic fluid or vomitus (6–8). Because the method provides quantitative assessment of the fraction of each hemoglobin species present, it is possible to determine relative contributions of maternal and fetal blood in situations of mixed blood sources; this determination is aided by the ease of quantifying HbF percentages in the blood of the mother and the newborn.

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References